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(54) Title: VECTORS DERIVED FROM SOUTH AFRICAN ARBOVIRUS NO. 86

(57) Abstract: Provided herein are alphavirus vectors derived from South African Arbovirus No. 86 (S.A.AR86) comprising attenuating mutations and methods of making the same. Also provided are improved viral vectors and helper constructs comprising a S.A.AR86 capsid enhancer sequence. The present invention also provides S.A.AR86 replicon and helper constructs comprising an alphavirus capsid enhancer sequence. Further provided are methods of administering an alphavirus vector comprising a heterologous nucleotide sequence (preferably encoding an immunogen or a therapeutic polypeptide) according to the invention to a cell or subject. In preferred embodiments, the alphavirus vector delivers the heterologous nucleotide sequence to the cells of the bone, bone marrow, and/or bone-associated connective tissue.

# Vectors derived from South African Arbovirus No. 86

## Statement of Federal Support

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## Field of the Invention

The present invention relates to alphavirus vectors and methods of making and administering the same, in particular, the present invention relates to alphavirus vectors derived from South African Arbovirus No. 86, and methods of making an administering the same.

## Background of the Invention

Several old world alphaviruses, including the Sindbis-group viruses, Ross River virus, O'nyong-nyong and Chikungunya, are associated with outbreaks of acute and persistent arthritis/arthralgia in humans (reviewed in Johnston, R. E. and C. J. Peters. 1996. Alphaviruses, p. 843-898. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Fields Virology. Lippincott-Raven, Philadelphia). Chikungunya and O'nyong-nyong have caused massive epidemics of acute, debilitating arthralgia in Africa and Asia (*Id.*). Ross River virus, also known as epidemic polyarthritis, is endemic to Australia (Aaskov et al., (1985) *Aust. J. Exp. Biol. Med. Sci.* **5**:587; Johnston, R. E. and C. J. Peters. 1996. Alphaviruses, p. 843-898. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Fields Virology. Lippincott-Raven, Philadelphia; Tai et al., (1993) *Med. J. Aust.* **158**:522), and caused a major epidemic that swept the South Pacific Islands in 1979, affecting 50,000 people on the Island of Fiji

(Aaskov et al., (1981) *Am. J. Trop. Med. Hyg.* **30**:1053). Sindbis-group alphaviruses, including Ockelbo, Karelian fever virus, and GirdwoodS.A. are associated with acute and persistent arthralgia in Northern Europe and South Africa (Malherbe et al., (1963) *S. Afr. Med. J.* **37**:547; Skogh et al., (1982) *Lancet* i:795; Tesh et al., (1982) *Ann. Rev. Med.* **33**:31). Ockelbo disease; one of the best characterized of the Sindbis-group alphavirus arthralgias, is often incapacitating (Johnston, R. E. and C. J. Peters. 1996. Alphaviruses, p. 843-898. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Fields Virology. Lippincott-Raven, Philadelphia; Tesh et al., (1982) *Ann. Rev. Med.* **33**:31), and one study found that symptoms lasted for months to years in 31% of patients (Niklasson et al., (1986) *Lancet* **1**:1039). Symptoms include arthralgia in one or more joints, including large joints such as the knee, hip, and elbow (reviewed in Johnston, R. E. and C. J. Peters. 1996. Alphaviruses, p. 843-898. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Fields Virology. Lippincott-Raven, Philadelphia; Tesh et al., (1982) *Ann. Rev. Med.* **33**:31). Pain within or around tendons is also a common trait of Sindbis-group virus infections (Tesh et al., (1982) *Ann. Rev. Med.* **33**:31). Rubella virus, another member of the family *Togaviridae* that is distantly related to the alphaviruses, is also associated with acute and persistent arthritis in humans (reviewed in Wolinsky, J. S. 1996. Rubella, p. 899-929. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Field's Virology. Lippencott-Raven, Philadelphia).

Mechanisms underlying Togavirus induced arthralgia/arthritis are not clearly understood, though direct viral replication within or around the affected joints may contribute to disease (reviewed in Tesh et al., (1982) *Ann. Rev. Med.* **33**:31). Ross River virus antigen has been detected in cell aspirates from the joints of acutely infected individuals (Fraser et al., (1983) *J. Clin. Path.* **36**:1256). Furthermore, patients suffering from persistent arthralgia following Ockelbo virus infection often have high levels of Ockelbo virus specific IgM, which suggests that the virus may persistently infect these individuals (Niklasson et al., (1988) *J. Infect. Dis.* **157**:832).

Understanding of the mechanisms leading to alphavirus mediated arthritis/arthralgia in humans has been hampered by the lack of a small animal model. Sindbis-group alphaviruses, Semliki Forrest virus, and Ross River virus replicate in bone associated connective tissue in neonatal mice, however skin and muscle are also major sites of viral replication (Klimstra et al., (1999) *J. Virol.* **73**:10387; Murphy et al., (1970) *Lab. Invest.* **22**:318; Murphy et al., (1973) *Journal of Infectious Disease* **127**:129; Trgovcich et al., (1996) *Virol.* **224**:73). Furthermore, infection of neonatal animals with these viruses results in rapidly fatal disease (*Id.*). This generalized pattern of replication and lethal outcome in neonatal mice has limited their usefulness as a model of bone and/or joint replication by arthralgia associated alphaviruses.

The present invention addresses a need in the art for improved alphavirus vectors and methods of administering the same.

## Summary of the Invention

The present invention is based, in part, on the discovery of improved reagents derived from South African Arbovirus No. 86 (S.A.AR86). Based on sequence comparisons, S.A.AR86 is in a subgroup of the Sindbis-group viruses that also includes GirdwoodS.A. and Ockelbo. Of these viruses, only S.A.AR86 is neurovirulent in weanling and adult mice, causing 90% to 100% mortality in mice of any age. The complete genomic sequence of S.A.AR86 is available (*see* Simpson et al., (1996) *Virology* **222**:464; U.S. Patent No. 5,639,650; U.S. Patent No. 5,811,407; GenBank accession number I46902; the disclosures of which are incorporated herein by reference in their entireties).

It has previously been reported that alphavirus vectors, including S.A.AR86 vectors, infect and exhibit long-term persistence in the cells of the bone marrow (*e.g.* osteoblasts), in particular, the cells of the endosteum, more particularly, endosteum cells within synovial joints. As one aspect, the present investigations have further characterized the targeting, expression and persistence of vectors derived from S.A.AR86.

S.A.AR86 infects bone cells, as well as cells of the bone marrow and bone-associated connective tissue. In particular, S.A.AR86 infects the cells of the periosteum, endosteum and tendons, generally within the epiphyses of the long bones adjacent to joints. Osteoblasts, among other cells, are targeted by S.A.AR86 in the periosteum and endosteum, whereas the target cells in tendons appear to be fibroblasts.

Cells that may be infected by the alphavirus vectors of the present invention include, but are not limited to, polymorphonuclear cells, hemopoietic stem cells (including megakaryocyte colony forming units (CFU-M), spleen colony forming units (CFU-S), erythroid colony forming units (CFU-E), erythroid burst forming units (BFU-E), and colony forming units in culture (CFU-C)), erythrocytes, macrophages (including reticular cells), monocytes, granulocytes, megakaryoctyes, lymphocytes, fibroblasts, osteoprogenitor cells, osteoblasts, osteoclasts, marrow stromal cells, chondrocytes and other cells of synovial joints.

The persistence of S.A.AR86 vectors, and other alphavirus vectors as disclosed herein and U.S. Patent No. 5,811,407, in the cells of the bone, bone marrow, and bone-associated connective tissue may advantageously be employed in methods of producing an immune response and/or for methods of therapeutic gene delivery to the cells of the bone and bone marrow, as well as bone-associated connective tissue and neurons. Preferably, the alphavirus vector persists at detectable levels within the cell for at least about one month, at least about two months, at least about three months, at least about four months, at least about six months, at least about nine months, at least about twelve months, or longer. More preferably, persistence at detectable levels is for a period of at least about three months.

As a further aspect, the present invention provides a S.A.AR86 genomic RNA, said S.A.AR86 genomic RNA comprising: (a) S.A.AR86 nonstructural protein (nsp) coding sequences, wherein the S.A.AR86 nonstructural protein coding sequences encode an attenuating mutation selected from the group consisting of:

(i) an attenuating mutation in the cleavage domain between the nsp1 and nsp2 coding sequences;

(ii) an attenuating mutation that results in a termination codon at nsp3 amino acid position 537,

(iii) an attenuating mutation comprising a substitution mutation at nsP3 amino acid position 385,

(iv) an attenuating mutation comprising an insertion of at least 8 amino acids following nsP3 amino acid position 385,

(v) a combination of the attenuating mutations of (i) to (iv),

and (b) a heterologous nucleotide sequence. In particular embodiments, the genomic RNA further comprises an alphavirus capsid enhancer sequence operatively associated with the heterologous nucleotide sequence.

As another aspect, the present invention provides a S.A.AR86 genomic RNA comprising (a) an alphavirus capsid enhancer sequence, and (b) a heterologous nucleotide sequence. In embodiments of the invention, the alphavirus capsid enhancer sequence is operably associated with the heterologous nucleotide sequence, so that expression of the heterologous nucleotide sequence is enhanced as compared to the level of expression in the absence of the capsid enhancer sequence.

As a further aspect, the present invention provides an infectious alphavirus particle comprising: (a) assembled alphavirus structural proteins, and (b) a S.A.AR86 genomic RNA according to the invention packaged within the assembled alphavirus structural proteins.

Also provided a compositions and pharmaceutical formulations comprising a plurality of the alphavirus particles of the invention.

As a still further aspect, the present invention provides a method of introducing a nucleotide sequence into a cell, comprising contacting a cell *in vitro* with an alphavirus particle according to the invention under conditions wherein the heterologous nucleotide sequence is introduced into the cell. The cell may be administered to a subject, *e.g.*, in *ex vivo* methods of gene

transfer, or an antigen presenting cell (such as a dendritic cell) may be administered to the subject to provide immunity.

As a yet further aspect, the present invention provides a method of administering a nucleotide sequence to a subject, comprising administering to the subject an alphavirus particle according to the present invention in a pharmaceutically acceptable carrier. In particular embodiments, the nucleotide sequence is administered in a method of producing an immune response.

The present invention also provides helper cells and methods for producing replicon particles according to the present invention.

As a further aspect, the present invention provides, a DNA molecule comprising: (a) a segment encoding a S.A.AR86 genomic RNA according to the invention, and (b) a promoter operatively associated with the segment encoding the S.A.AR86 genomic RNA. Also provided are infectious RNA transcripts encoded by the DNA molecule, vectors comprising the DNA vector, and cells comprising the vector.

These and other aspects of the invention are described in more detail in the description of the invention set forth below.

## Brief Description of the Drawings

**Figure 1.** S.A.AR86, as well as other Sindbis-group alphaviruses, replicates in bone/joint associated tissues. **A.** Six week old female CD-1 mice were infected with $10^3$ pfu of s55 i.v. Three days post infection, mice were sacrificed, exsanguinated, perfused with PBS, and femurs and quadriceps muscles removed by dissection and titered for infectious virus on BHK-21 cells. Data is shown as Log pfu/gram of tissue for femur and muscle, and as pfu/ml for serum. Each bar represents a single animal. The arrow indicates the limit of detection and asterisks denote samples below the limit of detection. Data shown is from one of four experiments. **B.** Six week old female CD-1 mice were infected i.v. with $10^3$ pfu of s51, sacrificed three days post-infection, and the right femur was removed for virus titration. Bone

marrow was aspirated from the diaphysis of the femur using 0.4 ml PBS/1%DCS per femur. Aspirates were freeze/thawed and titrated for infectious virus by plaque assay. Following marrow aspiration, the remaining femoral tissue was processed as in 1A and titrated for infectious virus by plaque assay. Titer is shown as total pfu per marrow aspirate or femur (without aspirated marrow) with each bar representing a single animal. The arrow indicates the limit of detection and asterisks denote samples with titers below the limit of detection. Shown is one of three comparable experiments. **C.** Six week old female CD-1 mice were infected iv with $10^3$ pfu of the viruses s55, TR339, or TRSB. Three days post infection mice were sacrificed, and both femurs removed for virus titration. Femurs were processed and titrated for infectious virus as in 1A. n = 3 mice/group. The arrow indicates the limit of detection. N/D = not done. Shown is one of two comparable experiments.

**Figure 2.** S.A.AR86 replication within the bone associated connective tissue on the epiphyses (ends) of the long bones. Four to six week old female CD-1 mice were infected iv with $10^3$ or $2.5x10^5$ pfu of s51 or mock infected. Mice were sacrificed at 24 or 48 hours post infection. Following decalcification, 5 uM thick paraffin embedded limb sections were probed with $^{35}$S-labeled riboprobes specific for S.A.AR86 or influenza strain PR/8 HA. **A.** S.A.AR86 specific *in situ* signal in periosteum of the tibia 24 hours after infection with $2.5 \times 10^5$ pfu of s51 iv. Muscle is designated by (M), while bone is designated by (B). **B.** Adjacent section probed with influenza specific riboprobe. **C.** Adjacent section, H + E staining. **D.** S.A.AR86 specific *in situ* signal in tendon and periosteum of the tibia proximal to knee joint 24 hours after infection with $2.5x10^5$ pfu of s51, iv. Tendon is designated by (T) and bone by (B). **E.** Adjacent section probed with influenza specific riboprobe. **F.** Adjacent section, H + E staining. Bar = 100 microns in each panel.

**Figure 3.** S.A.AR86-based double promoter viruses and replicons infect cells within the endosteum and periosteum. Four week old female CD-1

mice were infected iv with $10^3$ pfu of the double promoter virus s55-gfp(F); $1.5x10^6$ pfu of the double promoter virus s51-gfp or 2 x $10^6$ iu of the replicon REP91-gfp. Mock infected mice received PBS diluent alone. Mice infected with the $10^3$ dose of s55-gfp(F) were sacrificed three days post infection, while mice receiving high doses of s51-gfp or replicon were sacrificed at 12-14 hours post infection. Following sacrifice, mice were perfused with 4% paraformaldehyde, and hind limbs were decalcified before preparation of frozen sections. GFP positive cells were visualized by fluorescent microscopy. Panels A and B = $10^3$ pfu of s55-gfp(F), panels C and D = 1.5 x $10^6$ pfu of s51-gfp, and panels E and F = REP91-gfp. **Panels A and B**: GFP positive cells within the endosteum of a s55-gfp(F) infected mouse. 600x magnification, triple pass FITC/Texas Red filter. **Panel C:** GFP positive cells within the periosteum of a mouse infected with 1.5 x $10^6$ pfu of s51-gfp. Red staining indicates the presence of type I collagen in calcified tissue, which was identified using anti-mouse type I collagen (Rockland). 400x magnification, triple pass FITC/Texas Red filter. **Panel D:** GFP positive cell adjacent to calcified tissue in a s51-gfp infected mouse, 400x magnification, FITC filter. Panel E. GFP positive cell within the endosteum of REP91-gfp infected mouse, 400x magnification, Triple pass FITC/Texas Red filter. Panel F: GFP positive cells within the periosteum of a REP91-gfp infected mouse, 400 x magnification, Triple pass FITC/Texas Red filter. Tissue types are designated by the following abbreviations within the figures: B = bone, M = muscle, P = periosteum, and mc = marrow cavity.

**Figure 4.** *In vitro* translation reactions were performed using rabbit reticulocyte lysates to translate full length s55 (nsP1 538 Thr) and s51 (nsP1 538 Ile) RNAs. The translation reactions were performed in the presence of $^{35}S$ methionine so that the full-length translation products and their cleavage products could be visualized by gel electrophoresis. Following the initial radioactive pulse, the reactions were chased with excess nonradioactive

methionine and cycloheximide to stop new protein synthesis. Left panel: s55. Right panel: s51.

**Figure 5.** BHK-21 cells were infected with s55 (Thr) or s51 (Ile) at a multiplicity of infection of 5.0. Cells were labeled with $^{35}S$ methionine and then chased with an excess of cold methionine to measure the rate of nonstructural protein cleavage. At various times in the pulse/chase, cell lysates were generated and nsP1 was immunoprecipitated using protein specific antibodies. The radioactive proteins were visualized by gel electrophoresis and autoradiography.

**Figure 6.** BHK-21 cells were infected with s55 (nsP1 538 Thr) or s51 (nsP1 538 Ile) at a multiplicity of infection (MOI) of 5.0. Infection was allowed to go for one hour before washing the cells 3 times with room temperature PBS (1% DCS and $Ca^{++}$ /$Mg^{++}$). Cells were then incubated in growth media until time of RNA harvest. At 3 and 6 hours post infection, the dishes were transferred to ice and total cellular RNA was harvested. RNAse protection assays were performed to detect the viral 26S and plus strand RNAs (**Panel A**) or the minus strand RNA (**Panel B**). A probe specific for mouse ß-actin was used to control for RNA loading.

**Figure 7.** BHK-21 cells were inoculated with S.A.AR86 derived replicon particles with the wild type Thr or the attenuating Ile at nsP1 position 538. These replicons expressed green fluorescent protein (GFP) from their 26S promoters, which allowed analysis of GFP expression as a readout for 26S promoter expression. Infections were performed at a multiplicity of infection of 0.1 and cells were analyzed by flow cytometry to measure of GFP fluorescence at 4 hours post infection.

**Figure 8.** Female CD-1 mice were immunized with $10^4$ infectious Units of REP89HA (nsP1 538 Thr) or REP91HA (nsP1 538 Ile). Serum from the

inoculated mice was evaluated for anti-influenza virus hemagglutinin (HA) antibody at 12 weeks post inoculation using an ELISA specific for influenza virus HA. Results are shown as the reciprocal of the last dilution positive for anti-HA antibody. These results demonstrate that the presence of Ile at nsP1 538 results in consistently higher serum anti-HA antibody responses in the immunized mice compared to mice immunized with the wild type REP89HA replicon containing Thr at nsP1 538. Female CD-1 mice were immunized with $10^4$ infectious Units of REP89HA (nsP1 538 Thr) or REP91HA (nsP1 538 Ile). Serum from the inoculated mice was evaluated for anti-influenza virus hemagglutinin (HA) antibody at 12 weeks post inoculation using an ELISA specific for influenza virus HA. Results are shown as the reciprocal of the last dilution positive for anti-HA antibody. These results demonstrate that the presence of Ile at nsP1 538 results in consistently higher serum anti-HA antibody responses in the immunized mice compared to mice immunized with the wild type REP89HA replicon containing Thr at nsP1 538.

## Detailed Description of the Invention

Except as otherwise indicated, standard methods known to those skilled in the art may be used for the construction and use of recombinant nucleotide sequences, vectors, helper constructs, transformed host cells, selectable markers, alphavirus vectors, viral infection of cells, production of attenuated viruses, and the like. Such techniques are known to those skilled in the art. *See, e.g.*, SAMBROOK *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed. (Cold Spring Harbor, NY, 1989); F. M. AUSUBEL *et al.* CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used in the description of the invention and the appended claims, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

As used herein, the term "polypeptide" encompasses both peptides and proteins.

The term "alphavirus" has its conventional meaning in the art, and includes Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Everglades virus, Mucambo virus, Pixuna virus, Western Encephalitis virus (WEE), Sindbis virus, South African Arbovirus No. 86 (S.A.AR86), Girdwood S.A. virus, Ockelbo virus, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'Nyong-Nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzlagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, Buggy Creek virus, and any other virus classified by the International Committee on Taxonomy of Viruses (ICTV) as an alphavirus.

Preferred alphaviruses for use in the present invention are Sindbis virus strains (*e.g.*, TR339), S.A.AR86 virus, Girdwood S.A. virus, and Ockelbo virus, still more preferably, S.A.AR86, and chimeric viruses thereof.

An "Old World alphavirus" is a virus that is primarily distributed throughout the Old World. Alternately stated, an Old World alphavirus is a virus that is primarily distributed throughout Africa, Asia, Australia and New Zealand, or Europe. Exemplary Old World viruses include SF group alphaviruses and SIN group alphaviruses. SF group alphaviruses include Semliki Forest virus, Middelburg virus, Chikungunya virus, O'Nyong-Nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, and Una virus. SIN group alphaviruses include Sindbis virus, South African Arbovirus No. 86, Ockelbo virus, Girdwood S.A. virus, Aura virus, Whataroa virus, Babanki virus, and Kyzylagach virus.

The phrase "alphavirus structural protein(s)" as used herein refers to one or more of the proteins that are required to produce a functional alphavirus particle that encapsidates the alphavirus genomic RNA. The alphavirus structural proteins include the capsid protein, E1 glycoprotein, E2 glycoprotein, E3 protein and 6K protein. The alphavirus particle comprises the alphavirus

structural proteins assembled to form an enveloped nucleocapsid structure. As known in the art, alphavirus structural subunits consisting of a single viral protein, capsid, associate with themselves and with the RNA genome to form the icosahedral nucleocapsid, which is then surrounded by a lipid envelope covered with a regular array of transmembranal protein spikes, each of which consists of a heterodimeric complex of two glycoproteins, E1 and E2 (*See* Paredes et al., (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9095-99; Paredes et al., (1993) *Virology* **187**, 324-32; Pedersen et al., (1974) *J. Virol.* **14**:40).

An alphavirus "genomic RNA" indicates the alphavirus RNA transcript. The wild-type alphavirus genome is a single-stranded, messenger-sense RNA, modified at the 5'-end with a methylated cap, and at the 3'-end with a variable-length poly (A) tract. The viral genome is divided into two regions: the first encodes the nonstructural or replicase proteins (nsP1-nsP4) and the second encodes the viral structural proteins (Strauss and Strauss, *Microbiological Rev.* (1994) **58:**491-562). As used herein, the term "genomic RNA" encompasses recombinant alphavirus genomes (*e.g.*, containing a heterologous nucleotide sequence), viral genomes containing one or more attenuating mutations, deletions, insertions, and/or otherwise modified viral genomes. For example, the "genomic RNA" may be modified to form a double-promoter molecule or a replicon (each as described below).

A "chimeric" alphavirus as used herein comprises the alphavirus structural proteins and a genomic RNA from another alphavirus. In embodiments of the invention, the chimeric alphavirus comprises a S.A.AR86 genomic RNA and alphavirus structural proteins from another alphavirus (*e.g.*, Sindbis, GirdwoodS.A., Ockelbo, and the like). In other embodiments of the invention, the chimeric alphavirus comprises S.A.AR86 alphavirus structural proteins and a genomic RNA from another alphavirus (*e.g.*, Sindbis, GirdwoodS.A., Ockelbo, and the like). In particular embodiments, the alphavirus structural proteins comprise structural proteins from two or more alphaviruses.

An "infectious" alphavirus particle is one that can introduce the alphavirus genomic RNA into a permissive cell, typically by viral transduction. Upon introduction into the target cell, the genomic RNA serves as a template for RNA transcription (*i.e.*, gene expression). The "infectious" alphavirus particle may be "replication-competent" (*i.e.*, can transcribe and replicate the alphavirus genomic RNA) and "propagation-competent" (*i.e.*, results in a productive infection in which new alphavirus particles are produced). In embodiments of the invention, the "infectious" alphavirus particle is a replicon particle (as described below) that may introduce the genomic RNA (*i.e.*, replicon) into a host cell, is "replication-competent" to replicate the genomic RNA, but is "propagation-defective" in that it is unable to produce new alphavirus particles in the absence of helper sequences that complement the deletions or other mutations in the replicon (*i.e.*, provide the structural proteins that are not provided by the replicon).

As used herein, an "isolated" nucleic acid (*e.g.*, an "isolated DNA" or an "isolated genomic RNA") means a nucleic acid separated or substantially free from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the nucleic acid.

Likewise, an "isolated" polypeptide means a polypeptide that is separated or substantially free from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polypeptide.

As used herein, the terms "deleted" or "deletion" mean either total deletion of the specified segment or the deletion of a sufficient portion of the specified segment to render the segment inoperative or nonfunctional, in accordance with standard usage *See, e.g.,* United States Patent No. 4,650,764 to Temin et al.).

I. Alphavirus Vectors.

The present invention is practiced using alphavirus vectors, more preferably a propagation-incompetent alphavirus vector, still more preferably an alphavirus replicon vector (as described below). Alphavirus and replicon vectors are described in U.S. Patent No. 5,505,947 to Johnston et al.; U.S. Patent No. 5,792,462 to Johnston et al., U.S. Patent No. 5,814,482 to Dubensky et al., U.S. Patent No. 5,843,723 to Dubensky et al., U.S. Patent No. 5,789,245 to Dubensky et al., U.S. Patent No. 5,739,026 to Garoff et al., the disclosures of which are incorporated herein by reference in their entireties. More preferably, the alphavirus vector is a S.A.AR86 vector, a S.A.AR86 replicon vector, or S.A.AR86 chimeric vector comprising a S.A.AR86 genomic RNA.

Alphavirus vectors elicit a strong host response to immunogen. While not wishing to be held to any particular theory of the invention, it appears that alphavirus vectors induce a more balanced and comprehensive immune response (*i.e.*, cellular and humoral immunity) than do conventional vaccination methods. Moreover, it appears that alphavirus vectors induce a strong immune response, in part, because they directly infect and replicate within dendritic cells. The resulting presentation of antigen to the immune system induces a strong immune response. The alphavirus 26S subgenomic promoter also appears to give high level of expression of a heterologous nucleic acid encoding antigen.

The alphavirus vector preparation may be partially or highly purified, or may be a relatively crude cell lysate or supernate from a cell culture, as known in the art.

The alphavirus vector may be a chimeric alphavirus, as that term is understood in the art. For example, the alphavirus structural proteins may be from one alphavirus (preferably, S.A.AR86) and the nucleic acid packaged within the capsid may be from another alphavirus. Alternatively, the alphavirus virus may be assembled from structural proteins derived from more than one alphavirus.

A. Double Promoter Vectors.

In one embodiment of the invention, the alphavirus genomic RNA is a double promoter. A double promoter vector is a replication and propagation competent virus. Double promoter vectors are described in United States Patent No. 5,185,440, 5,505,947 and 5,639,650, the disclosures of which are incorporated in its entirety by reference. Preferred alphaviruses for constructing the double promoter vectors are S.A.AR86, Girdwood S.A., TR339 and Ockelbo viruses. Exemplary double promoters are derived from a S.A.AR86 genomic RNA. More preferably, the double promoter vector contains one or more attenuating mutations. Attenuating mutations are described in more detail hereinbelow.

In preferred embodiments, the double promoter vector is constructed so as to contain a second subgenomic promoter (*i.e.*, 26S promoter) inserted 3' to the virus RNA encoding the structural proteins. The heterologous RNA is inserted between the second subgenomic promoter, so as to be operatively associated therewith, and the 3' UTR of the virus genome. Heterologous RNA sequences of less than 3 kilobases, more preferably those less than 2 kilobases, and more preferably still those less than 1 kilobase, can be inserted into the double promoter vector. In a preferred embodiment of the invention, the double promoter vector is derived from a Girdwood S.A. genomic RNA, and the second subgenomic promoter is a duplicate of the Girdwood S.A. subgenomic promoter. In an alternate preferred embodiment, the double promoter vector is derived from a Sindbis (*e.g.*, TR339) genomic RNA, and the second subgenomic promoter is a duplicate of the TR339 subgenomic promoter. In another preferred embodiment, the double promoter vector is derived from a S.A.AR86 genomic RNA, and the second subgenomic promoter is a duplicate of the S.A.AR86 subgenomic promoter.

B. Replicon Vectors.

Replicon vectors, which are infectious, propagation-defective, virus vectors can also be used to carry out the present invention. Replicon vectors are described in more detail in WO 96/37616 to Johnston et al., U.S. Patent No. 5,505,947 to Johnston et al., and U.S. Patent No. 5,792,462 to Johnston et al. Preferred alphaviruses for constructing the replicon vectors according to the present invention are S.A.AR86, Girdwood S.A., Sindbis (*e.g.*, TR339), and Ockelbo, and chimeras thereof.

In general, in the replicon system, a foreign gene to be expressed is inserted in place of at least a portion of one or more of the viral structural protein genes in a transcription vector containing the viral sequences necessary for viral replication (*e.g.*, the nsp1-4 genes). RNA transcribed from this vector contains sufficient viral sequences (*e.g.*, the viral nonstructural genes) responsible for RNA replication and transcription. Thus, if the transcribed RNA is transfected into susceptible cells, it will be replicated and translated to give the replication proteins. These proteins will transcribe the transfected RNA, including the transgene, which will then be translated to produce high levels of the foreign protein. The autonomously replicating RNA (*i.e.*, replicon) can only be packaged into virus particles if the deleted alphavirus structural protein genes are provided on one or more helper molecules, which are provided to the helper cell.

Preferably, the helper molecules do not contain the viral nonstructural genes for replication, but these functions are provided *in trans* by the replicon molecule. The transcriptase functions translated from the replicon molecule transcribe the structural protein genes on the helper molecule, resulting in the synthesis of viral structural proteins and packaging of the replicon into virus-like particles. As the alphavirus packaging or encapsidation signal is located within the nonstructural genes, the absence of these sequences in the helper molecules precludes their incorporation into virus particles.

Accordingly, the replicon molecule is "propagation defective," as described hereinabove. The resulting alphavirus particles are propagation

defective inasmuch as the replicon RNA in these particles does not include all of the alphavirus structural proteins required for encapsidation, at least a portion of at least one of the required structural proteins being deleted therefrom, such that the replicon RNA initiates only an abortive infection; no new viral particles are produced, and there is no spread of the infection to other cells.

Typically, the replicon molecule comprises an alphavirus packaging signal.

The replicon molecule is self-replicating. Accordingly, the replicon molecule comprises sufficient coding sequences for the alphavirus nonstructural polyprotein so as to support self-replication. In embodiments of the invention, the replicon encodes the alphavirus nsP1, nsP2, nsP3 and nsP4 proteins.

The replicon molecules of the invention "do not encode" one or more of the alphavirus structural proteins. By "do(es) not encode" one or more structural proteins, it is intended that the replicon molecule does not encode a functional form of the one or more structural proteins and, thus, a complementing sequence must be provided by a helper or packaging cell to produce new virus particles. In embodiments of the invention, the replicon molecule does not encode any of the alphavirus structural proteins.

The replicon may not encode the structural protein(s) because the coding sequence is partially or entirely deleted from the replicon molecule. Alternatively, the coding sequence is otherwise mutated so that the replicon does not express the functional protein. In embodiments of the invention, the replicon lacks all or substantially all of the coding sequence of the structural protein(s) that is not encoded by the replicon, *e.g.*, so as to minimize recombination events with the helper sequences.

In particular embodiments, the replicon molecule may encode at least one, but not all, of the alphavirus structural proteins. For example, the alphavirus capsid protein may be encoded by the replicon molecule. Alternatively, one or both of the alphavirus glycoproteins may be encoded by the replicon molecule. As a further alternative, the replicon may encode the capsid protein and either the E1 or E2 glycoprotein.

In more preferred embodiments, none of the alphavirus structural proteins are encoded by the replicon molecule. For example, all or substantially all of the sequences encoding the alphavirus capsid protein and glycoproteins may be deleted from the replicon molecule.

Preferably, a composition comprising a population of replicon particles of the invention contains no detectable-replication competent alphavirus particles. Replication-competent virus may be detected by any method known in the art, *e.g.*, by neurovirulence following intracerebral injection into suckling mice, or by passage twice on alphavirus-permissive cells (*e.g.*, BHK cells) and evaluation for virus induced cytopathic effects.

**II. Attenuating Mutations.**

The present invention also provides alphavirus genomic RNA and particles (*e.g.*, S.A.AR86) including attenuating mutations. The phrases "attenuating mutation" and "attenuating amino acid," as used herein, mean a nucleotide sequence containing a mutation, or an amino acid encoded by a nucleotide sequence containing a mutation, which mutation results in a decreased probability of causing disease in its host (*i.e.*, reduction in virulence), in accordance with standard terminology in the art. *See, e.g.,* B. Davis et al., MICROBIOLOGY 132 (3d ed. 1980). The phrase "attenuating mutation" excludes mutations or combinations of mutations which would be lethal to the virus.

Appropriate attenuating mutations will be dependent upon the alphavirus used. Suitable attenuating mutations within the alphavirus genome will be known to those skilled in the art. Exemplary attenuating mutations include, but are not limited to, those described in United States Patent No. 5,505,947 to Johnston et al., U.S. Patent No. 5,185,440 to Johnston et al., U.S. Patent No. 5,643,576 to Davis et al., U.S. Patent No. 5,792,462 to Johnston et al., and U.S. Patent No. 5,639,650 to Johnston et al., the disclosures of which are incorporated herein in their entirety by reference.

When the alphavirus structural proteins are from VEE, suitable attenuating mutations may be selected from the group consisting of codons at E2 amino acid position 76 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 76; codons at E2 amino acid position 120 which specify an attenuating amino acid, preferably lysine as E2 amino acid 120; codons at E2 amino acid position 209 which specify an attenuating amino acid, preferably lysine, arginine or histidine as E2 amino acid 209; codons at E1 amino acid 272 which specify an attenuating amino acid, preferably threonine or serine as E1 amino acid 272; codons at E1 amino acid 81 which specify an attenuating amino acid, preferably isoleucine or leucine as E1 amino acid 81; codons at E1 amino acid 253 which specify an attenuating amino acid, preferably serine or threonine as E1 amino acid 253; or the deletion of E3 amino acids 56-69, or a combination of the deletion of E3 amino acids 56-59 together with codons at E1 amino acid 253 which specify an attenuating mutation, as provided above.

Also preferred are alphavirus vectors in which there is an attenuating mutation in the capsid protease that reduces, preferably ablates, the autoprotease activity of the capsid and results, therefore, in non-viable virus. Capsid mutations that reduce or ablate the autoprotease activity of the alphavirus capsid are known in the art, *see e.g.*, WO 96/37616 to Johnston et al., the disclosure of which is incorporated herein in its entirety. In particular embodiments, the alphavirus vector comprises a VEE capsid protein in which the capsid protease is ablated, *e.g.*, by introducing an amino acid substitution at VEE capsid position 152, 174, or 226. Alternatively, one or more of the homologous positions in other alphaviruses may be altered to reduce capsid protease activity.

If the alphavirus vector comprises a Sindbis-group virus (*e.g.*, Sindbis, S.A.AR86, GirdwoodSA, Ockelbo) capsid protein, the attenuating mutation may be a mutation at capsid amino acid position 215 (*e.g.*, a Ser→Ala) that reduces capsid autoprotease activity (*see*, Hahn et al., (1990) *J. Virology* **64**:3069).

In particular, preferred, embodiments, the "attenuating" mutation reduces (*e.g.*, by at least 25%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more) the neurovirulence of the alphavirus vector (*e.g.*, as determined by intracerebral injection in weanling or adult mice). In embodiments of the invention, the attenuated virus is an attenuated S.A.AR86 virus vector (or chimeric vector, *e.g.*, comprising a S.A.AR86 genomic RNA) with reduced neurovirulence.

It is not necessary that the attenuating mutations of the invention eliminate all pathology or adverse effects associated with virus administration, as long as there is some improvement or benefit (*e.g.*, increased safety and/or reduced morbidity and/or reduced mortality) as a result of the attenuating mutation.

In other embodiments of the invention, the attenuating mutation does not result in a significant reduction in transgene expression from the attenuating alphavirus genomic RNA, *i.e.*, transgene expression is essentially the same as in non-attenuated viruses. Alternatively, transgene expression may even be enhanced in the attenuated virus as compared with the non-attenuated virus.

When the alphavirus structural and/or non-structural proteins are from S.A.AR86, exemplary attenuating mutations in the structural and non-structural proteins include, but are not limited to, codons at nsP1 amino acid position 538 which specify an attenuating amino acid, preferably isoleucine as nsP1 amino acid 538; codons at E2 amino acid position 304 which specify an attenuating amino acid, preferably threonine as E2 amino acid 304; codons at E2 amino acid position 314 which specify an attenuating amino acid, preferably lysine as E2 amino acid 314; codons at E2 amino acid 372 which specify an attenuating amino acid, preferably leucine, at E2 amino acid residue 372; codons at E2 amino acid position 376 which specify an attenuating amino acid, preferably alanine as E2 amino acid 376; in combination, codons at E2 amino acid residues 304, 314, 372 and 376 which specify attenuating amino acids, as described above; codons at nsP2 amino acid position 96 which specify an attenuating amino acid, preferably glycine as nsP2 amino acid 96; and codons at nsP2 amino acid position 372 which specify an attenuating amino acid,

preferably valine as nsP2 amino acid 372; in combination, codons at nsP2 amino acid residues 96 and 372 which encode attenuating amino acids at nsP2 amino acid residues 96 and 372, as described above; codons at nsP2 amino acid residue 529 which specify an attenuating amino acid, preferably leucine, at nsP2 amino acid residue 529; codons at nsP2 amino acid residue 571 which specify an attenuating amino acid, preferably asparagine, at nsP2 amino acid residue 571; codons at nsP2 amino acid residue 682 which specify an attenuating amino acid, preferably arginine, at nsP2 amino acid residue 682; codons at nsP2 amino acid residue 804 which specify an attenuating amino acid, preferably arginine, at nsP2 amino acid residue 804; codons at nsp3 amino acid residue 22 which specify an attenuating amino acid, preferably arginine, at nsP3 amino acid residue 22; and in combination, codons at nsP2 amino acid residues 529, 571, 682 and 804 and at nsP3 amino acid residue 22 which specify attenuating amino acids, as described above.

In particular preferred embodiments, the attenuating mutation is an attenuating mutation in one or more of the cleavage domains between the alphavirus nonstructural (nsp) genes, *e.g.*, the nsP1/nsP2 cleavage region, the nsP2/nsP3 cleavage region, and/or the nsP3/nsP4 cleavage region. In embodiments of the invention, the attenuated virus includes an attenuating mutation in the nsP1/nsP2 cleavage region. Preferably, the attenuated virus comprises a S.A.AR86 genomic RNA comprising the attenuating mutation(s) in the nsp cleavage domain(s).

The cleavage regions between the alphavirus non-structural proteins are fairly well conserved and have been discussed in Strauss and Strauss, *Microbiological Rev.* **58**, 491-562, 494 (1994); the disclosure of which is incorporated herein in its entirety. In general, the amino acids in the cleavage domains have been designated, in the amino to carboxy terminus direction, as P4-P3-P2-P1 ↓ P1'-P2'-P3'-P4' (arrow indicates the site of protease cleavage). Preferably, the attenuating mutation is at the P3 position of the cleavage domain.

In other embodiments of the invention, the attenuating mutation is in the P4, P3, P1' or P2' positions of the nsP1/nsP2 cleavage domain, which positions are less highly conserved among the various alphaviruses and likely to better withstand alterations.

In other embodiments of the invention, the attenuating mutation is in the cleavage domain between the S.A.AR86 nonstructural proteins. Exemplary attenuating mutations include attenuating mutations in the nsP1/nsP2 cleavage domain at amino acids 534-547 of the S.A.AR86 nonstructural polyprotein P1234, attenuating mutations in the nsP1/nsP2 cleavage domain at amino acids 537-544 of the S.A.AR86 nonstructural polyprotein P1234, attenuating mutations in the nsP2/nsP3 cleavage domain at amino acids 1341-1354 of the S.A.AR86 nonstructural polyprotein P1234, attenuating mutations in the nsP2/nsP3 cleavage domain at amino acids 1344-1351 of the S.A.AR86 nonstructural polyprotein P1234, attenuating mutations in the nsP3/nsP4 cleavage domain at amino acids 1884-1897 of the S.A.AR86 nonstructural polyprotein P1234, and attenuating mutations in the nsP3/nsP4 cleavage domain at amino acids 1887-1894 of the S.A.AR86 nonstructural polyprotein P1234.

In embodiments of the invention, the attenuating mutation is in the cleavage domain between the nsP1 and nsP2 genes of S.A.AR86. An exemplary attenuating mutation is a mutation at S.A.AR86 nsP1 amino acid 538 (position P3), more preferably a substitution mutation at S.A.AR86 nsP1 amino acid 538, still more preferably a Thr→Ile substitution at S.A.AR86 nsP1 amino acid 538.

The present inventors have found that a Thr→Ile substitution at S.A.AR86 nsP1 amino acid 538 may be incorporated into alphavirus vectors comprising S.A.AR86 nonstructural proteins to advantageously increase transgene expression (at the RNA or protein level), *e.g.*, by at least about 2-fold, 5-fold, 10-fold or more, in particular, at early time-points post-infection (*e.g.*, from about 0.5 to about 6 hours post-infection). In embodiments of the invention, the transgene is operatively associated with the 26S subgenomic promoter.

While not wishing to be held to any particular theory of the invention, it appears that the Thr→Ile attenuating mutation at S.A.AR86 nsP1 amino acid 538 enhances (*i.e.*, increases) the rate of cleavage, thereby eliciting an augmented type I interferon response by the host, which may account for the attenuation. This view is supported by the finding that co-administration of attenuated and non-attenuated S.A.AR vectors results in attenuation of the non-attenuated virus.

Accordingly, the present invention encompasses other attenuating mutations in the nsp cleavage regions that result in an enhanced rate of cleavage and/or an enhanced type 1 interferon response. Methods of determining the rate of cleavage of the nsp polyprotein and/or interferon response may be by any method known in the art, including the methods disclosed herein (*see, e.g.*, the Examples).

Likewise, those skilled in the art may identify attenuating mutations other than those specifically disclosed herein using other methods known in the art, *e.g.*, looking at neurovirulence in weanling or adult mice following intracerebral injection.

To identify other attenuating mutations other than those specifically disclosed herein, amino acid substitutions may be based on any characteristic known in the art, including the relative similarity or differences of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like.

Amino acid substitutions other than those disclosed herein may be achieved by changing the codons of the genomic RNA sequence (or a DNA sequence), according to the following codon table:

TABLE 1

| Amino Acids | | | Codons | | | | | |
|---|---|---|---|---|---|---|---|---|
| Alanine | Ala | A | GCA | GCC | GCG | GCU | | |
| Cysteine | Cys | C | UGC | UGU | | | | |
| Aspartic acid | Asp | D | GAC | GAU | | | | |
| Glutamic acid | Glu | E | GAA | GAG | | | | |
| Phenylalanine | Phe | F | UUC | UUU | | | | |
| Glycine | Gly | G | GGA | GGC | GGG | GGU | | |
| Histidine | His | H | CAC | CAU | | | | |
| Isoleucine | Ile | I | AUA | AUC | AUU | | | |
| Lysine | Lys | K | AAA | AAG | | | | |
| Leucine | Leu | L | UUA | UUG | CUA | CUC | CUG | CUU |
| Methionine | Met | M | AUG | | | | | |
| Asparagine | Asn | N | AAC | AAU | | | | |
| Proline | Pro | P | CCA | CCC | CCG | CCU | | |
| Glutamine | Gln | Q | CAA | CAG | | | | |
| Arginine | Arg | R | AGA | AGG | CGA | CGC | CGG | CGU |
| Serine | Ser | S | AGC | ACU | UCA | UCC | UCG | UCU |
| Threonine | Thr | T | ACA | ACC | ACG | ACU | | |
| Valine | Val | V | GUA | GUC | GUG | GUU | | |
| Tryptophan | Trp | W | UGG | | | | | |
| Tyrosine | Tyr | Y | UAC | UAU | | | | |

In identifying other attenuating mutations, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (*see*, Kyte and Doolittle, (1982) *J. Mol. Biol.* **157**:105; incorporated herein by reference in its entirety). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure

of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, *Id.*), these are:

isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

Accordingly, the hydropathic index of the amino acid (or amino acid sequence) may be considered when identifying additional attenuating mutations according to the present invention.

It is also understood in the art that the substitution of amino acids can be made on the basis of hydrophilicity. U.S. Patent No. 4,554,101 (incorporated herein by reference in its entirety) states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (±3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); seine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± I); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine *(-2.5);* tryptophan (-3.4).

Thus, the hydrophilicity of the amino acid (or amino acid sequence) may be considered when identifying additional attenuating mutations according to the present invention.

Other illustrative attenuating mutations include an attenuating mutation at amino acid position 537 of the S.A.AR86 nsP3 protein, more preferably a substitution mutation at this position (as described above; *see* Table I), still

more preferably a nonsense mutation that results in substitution of a termination codon. Translational termination (*i.e.*, stop) codons are known in the art, and include the "opal" (UGA), "amber" (UAG) and "ochre" (UAA) termination codons. In embodiments of the invention, the attenuating mutation results in a Cys→opal substitution at S.A.AR85 nsP3 amino acid position 537.

Further exemplary attenuating mutations include an attenuating insertion mutation following amino acid 385 of the S.A.AR86 nsP3 protein. Preferably, the insertion comprises an insertion of at least 2, 4, 6, 8, 10, 12, 14, 16 or 20 amino acids. In embodiments of the invention, the inserted amino acid sequence is rich in serine and threonine residues (*e.g.*, comprises at least 2, 4, 6, or 8 such sites) that serve as a substrate for phosphorylation by serine/threonine kinases.

! Ça ! Ça? Çü Preferably, the attenuating mutation comprises an insertion of the amine